Chemistry & Biology
Article

Characterization of SyrC, an Aminoacyltransferase Shuttling Threonyl and Chlorothreonyl Residues in the Syringomycin Biosynthetic Assembly Line

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DOI 10.1016/j.chembiol.2006.11.005

SUMMARY

Syringomycin, a lipopeptidolactone assembled from nine amino acid monomers by four enzymes, SyrB1, SyrB2, SyrC, and SyrE, is a cyclic nonribosomal peptide made by plant-associated *Pseudomonas* spp. This assembly is unusual because the terminal residue, 4-chlorothreonine, has been proposed to be added in *trans* since the ninth module of the megasynthetase SyrE lacks an adenylation domain required for Thr/Cl-Thr activation. SyrC is now identified as a Thr/Cl-Thr aminoacyltransferase, shuttling the Thr/Cl-Thr moiety between the pantetheinyl arms of the thiolation domain of SyrB1 and the thiolation domain in module nine of SyrE. SyrC uses Cys224 as a catalytic nucleophile to generate a Thr/Cl-Thr-*S*-enzyme intermediate during transfer. SyrC joins a growing family of such aminoacyl-shuttling enzymes that also use covalent catalysis to move aminoacyl groups from carrier proteins during coumermycin and coronamic acid biosynthesis.

INTRODUCTION

The phytotoxic natural product syringomycin E is a cyclic lipodepsipeptide produced by *Pseudomonas syringae* pv. *syringae* [1, 2]. The nonapeptidolactone comprising the syringomycin scaffold contains unusual amino acids at 5 of the 9 residues, including D-diaminobutyric acid, L-diaminobutyric acid, dehydro-Thr, L-*threo*-OH-Asp, and 4-Cl-L-Thr (Figure 1A). The syringomycin gene cluster in *P. syringae* pv. *syringae* B301D contains four genes relevant to syringomycin biosynthesis, namely, *syrB1*, *syrB2*, *syrC*, and *syrE* [1]. The roles of SyrB1 and SyrB2 in the generation of the 4-Cl-L-Thr residue have been well characterized. SyrB1 is an adenylation-thiolation didomain enzyme that is responsible for activating and loading L-Thr. The non-heme Fe^{II} halogenase SyrB2 chlorinates L-Thr when it is tethered to the thiolation domain of SyrB1, thereby generating the tethered 4-Cl-L-Thr moiety that is later incorporated into the syringomycin nonapeptide [3]. SyrE is an NRPS megasynthetase composed of nine modules (Figure 1B), the first eight of which contain a condensation, adenylation, and thiolation domain. In contrast, the ninth module of SyrE lacks an adenylation domain, suggesting that the final amino acid, 4-Cl-Thr_9, must be loaded onto the ninth T domain in *trans*.

SyrC belongs to the α/β-hydrolase superfamily and shows homology to a small group of acyltransferases that have recently been shown to be involved in other NRPS and PKS systems [4, 5]. Prior efforts to identify the role of SyrC in syringomycin biosynthesis have suggested that the enzyme may be capable of hydrolyzing the CoA moiety from long-chain fatty acids, including 3-hydroxydodecanoyl-CoA, the lipid attached to the N-terminal serine of syringomycin [6]. However, no evidence for that role has been forthcoming. Instead, we report here that SyrC is an aminoacyltransferase, shuttling the threonyl moiety in *trans* between the thiolation domain of SyrB1 and SyrE, setting up the final elongation to the full-length nonapeptidyl chain.

RESULTS

Cloning and Expression of SyrB1, SyrC, SyrC C224A, and $SyrE_{8,9}$ Constructs

The genes encoding the 66 kDa SyrB1 and 44 kDa SyrC were amplified from the *Pseudomonas syringae* pv. *syringae* B301D gene cluster and were cloned into N-terminally His_6-tagged expression vectors. The SyrE-$A_8T_8C_9T_9TE$ 160 kDa five-domain fragment (hereafter termed $SyrE_{8,9}$) of the 28 kbp *syrE* gene was amplified and cloned in the same manner. The point mutant SyrC C224A was generated by SOE mutagenesis of the SyrC construct described above and was cloned into an N-terminally His_6-tagged expression vector. The expression vectors for all of the constructs mentioned above were transformed into *E. coli*, and the proteins were expressed at 15°C after induction of the cultures with 0.1 mM IPTG. All proteins were purified by nickel-affinity chromatography (Figure 1C). Yields of 10–15 mg protein/l culture were obtained.

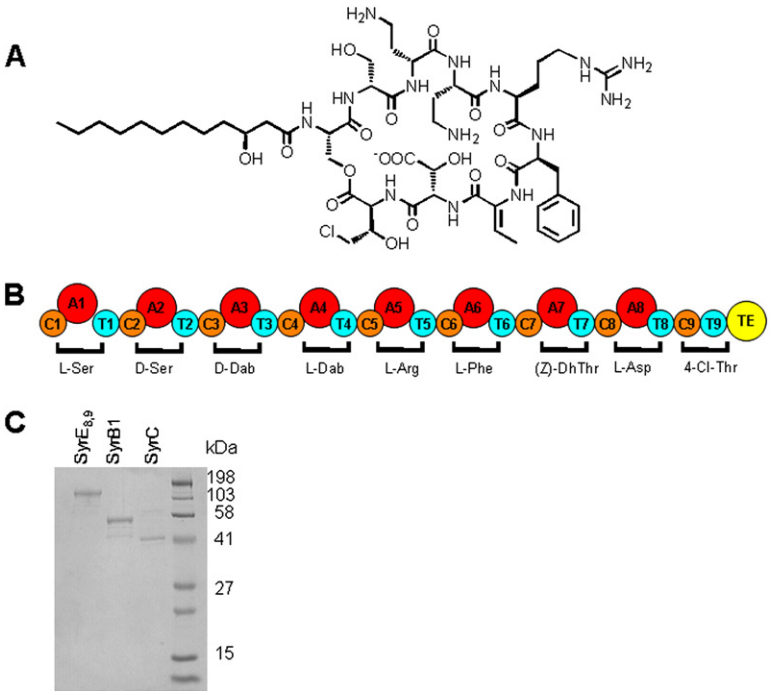

Figure 1. Syringomycin E Structure and the Proteins Involved in Its Biosynthesis
(A) Syringomycin E structure.
(B) SyrE module organization.
(C) SDS polyacrylamide gel of purified Syr proteins.

Several errors were found when comparing the cloned sequences of $SyrE_{8,9}$ and SyrC to the published sequences [1]. Multiple clones from different PCR reactions were sequenced to confirm that the errors did not result from errors incurred during PCR amplification. The correct sequences are shown in Figure S1 (see the Supplemental Data available with this article online). Similar types of errors were found in SyrB1 and SyrB2 by the original group that published the sequences, but these errors have since been corrected. The initial errors were simply due to the poor quality of the initial sequencing results, not to a difference in strains.

Demonstration of L-[^{14}C]Thr Transfer from SyrB1 to SyrC; Formation of a SyrC Acyl-Enzyme Intermediate

Prior work has shown that SyrB1 is the adenylation-thiolation (A-T) didomain protein that activates and then covalently tethers L-Thr in thioester linkage to the phosphopantetheinyl arm in the thiolation domain, where it then undergoes halogenation to produce 4-Cl-Thr-*S*-SyrB1 [3]. However, the multimodular syringomycin synthetase SyrE lacks a ninth adenylation domain to load the final 4-Cl-Thr residue onto its ninth thiolation domain (T_9) (Figure 1B). Therefore, we propose that SyrC transfers 4-Cl-Thr from SyrB1 to SyrE. To test the hypothesis that SyrC may engage in two-step aminoacyl transfer via a Thr/Cl-Thr-enzyme intermediate, the transfer of L-[^{14}C]Thr from [^{14}C]Thr-*S*-SyrB1 to SyrC was evaluated (Figure 4B, first half reaction). Although 4-Cl-Thr is the form of Thr incorporated into the syringomycin scaffold, we used L-[^{14}C]Thr to test for SyrC acyltransferase activity since it is readily available and can be detected by autoradiography, and since Thr_9 variants of syringomycin are known [7].

Recombinant SyrC was incubated with phosphopantetheinylated SyrB1 (holo-SyrB1) in the presence of L-[^{14}C]Thr and ATP in HEPES buffer. Aliquots of the reaction were quenched in SDS-PAGE loading buffer at various time points, and the proteins were subjected to SDS-PAGE. The autoradiogram of the gel revealed a detectable amount of radiolabel accumulating on SyrC in a time-dependent fashion (Figure 2A), suggesting the formation of an acyl-enzyme intermediate. This intermediate is only apparent in the absence of reducing agents, such as β-mercaptoethanol and dithiothreitol, indicating that it is quite labile to thiol nucleophiles.

Demonstration of SyrC-Mediated Transfer of L-[^{14}C]Thr from SyrB1 to SyrE

To determine whether SyrC can mediate L-[^{14}C]Thr transfer from SyrB1 to SyrE, we used an equivalent gel-based assay as described above, but we now monitored accumulation of the radiolabeled $SyrE_{8,9}$ fragment. Both SyrB1 and $SyrE_{8,9}$ were preincubated with the phosphopantetheinyltransferase Sfp [8, 9] to convert inactive apo forms of the thiolation domains to the HS-pantetheinyl-containing holo forms. $SyrE_{8,9}$ has two T domains that must be posttranslationally primed. SyrC was incubated with holo-SyrB1 and holo-$SyrE_{8,9}$ in the presence of L-[^{14}C]Thr and ATP in HEPES buffer. A build-up of radiolabel on SyrE was detectable over a 1 hr time period, showing that L-[^{14}C]Thr is indeed transferred from SyrB1 to SyrE. Control experiments verified that the transfer was both time and SyrC dependent (Figures 3A and 3B). In the absence of reducing agents, the SyrC acyl-enzyme intermediate is visible (Figure 3C).

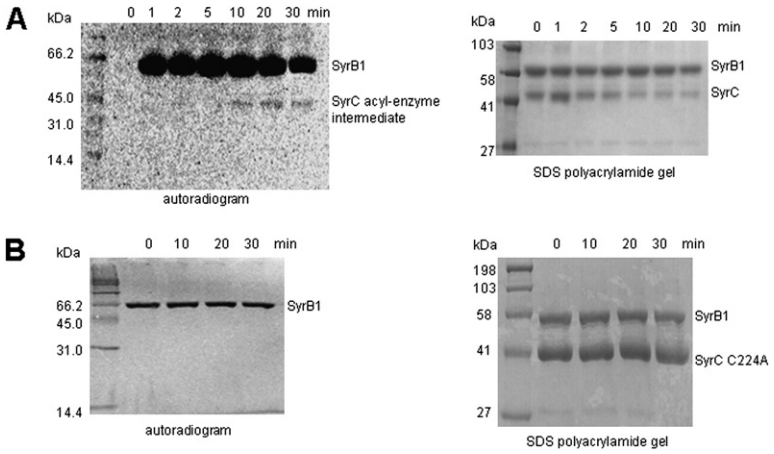

Figure 2. Assays for Transfer of L-[^{14}C]Thr from SyrB1 to SyrC and Acyl-Enzyme Intermediate Formation

(A) Autoradiogram and SDS polyacrylamide gel of the transfer assay with SyrC under nonreducing conditions during gel sample preparation.

(B) Autoradiogram and SDS polyacrylamide gel of the transfer assay with SyrC C224A under nonreducing conditions during gel sample preparation.

Identification of the Catalytic Residue Necessary for Acyltransferase Activity

Sequence comparison to other members of the α/β-hydrolase protein superfamily, notably CmaE [4], CouN7, and ClоN7 [5], suggested that the thiolate side chain of Cys224 could be the catalytic nucleophile; thus, the corresponding SyrC C224A mutant was constructed, expressed, and purified (Figure S2). The SyrC C224A mutant protein did not become detectably radiolabeled, suggesting that the point mutation prevents formation of the acyl-enzyme intermediate (Figures 2B and 4A). Therefore, given the first half reaction (Thr-*S*-SyrB1 to SyrC) and the overall reaction (Thr-*S*-SyrB1 to $SyrE_{8,9}$), we conclude that SyrC is competent to catalyze the second half reaction of the scheme depicted in Figure 4B (Thr-*S*-SyrC to $SyrE_{8,9}$). The migrating aminoacyl moiety is shuttling between thiol nucleophiles, Cys224 in SyrC, and the HS-pantetheinyl arms of the T domains in SyrB1 and SyrE.

Characterization of SyrC Amino Acid and Acceptor T Domain Specificity

Testing the selectivity of SyrC for transfer of amino acids other than L-Thr onto $SyrE_{8,9}$ required the use of alternate aminoacyl-*S*-SyrB1 proteins as donor substrates. From our prior studies, SyrB1 has very high specificity for loading L-Thr and cannot load alternate amino acids to any significant extent [3]. Therefore, instead of using SyrB1 as the donor T domain, we turned to a comparable free-standing A-T didomain enzyme, CmaA from the coronamic acid biosynthetic pathway [4]. Although CmaA normally activates and tethers the *allo* diastereomer of L-isoleucine on its T domain, CmaA is also capable of loading L-valine, which is available in radioactive form.

When SyrC was incubated with holo-CmaA and $SyrE_{8,9}$ in the presence of L-[^{14}C]Val and ATP in HEPES buffer and the transfer reaction was monitored by autoradiography (Figure 5), the build-up of radioactive label on SyrE in

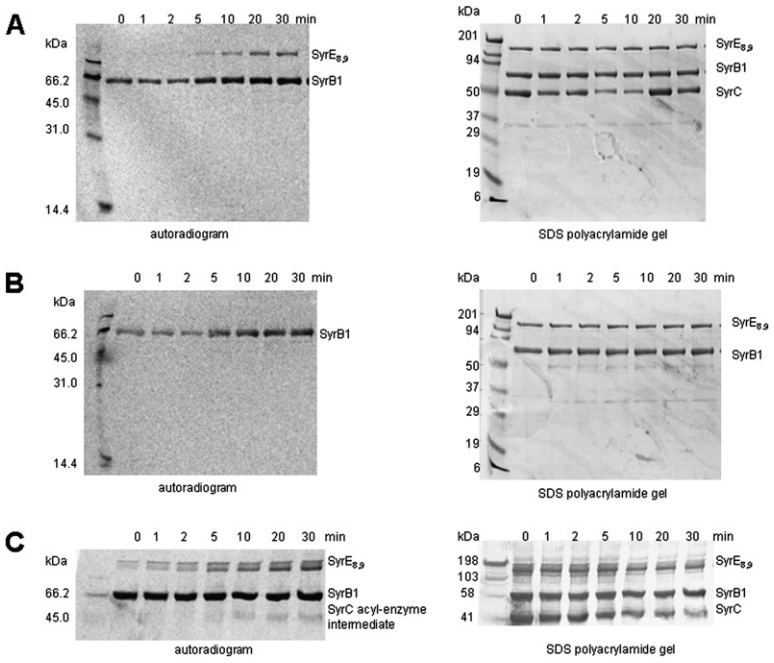

Figure 3. Assays for SyrC-Mediated Transfer of L-[^{14}C]Thr from SyrB1 to SyrE

(A) Autoradiogram and SDS polyacrylamide gel of the transfer assay with SyrC.

(B) Autoradiogram and SDS polyacrylamide gel of the transfer assay control without SyrC.

(C) Autoradiogram and SDS polyacrylamide gel of the transfer assay under nonreducing conditions during gel sample preparation, showing formation of the acyl-enzyme intermediate.

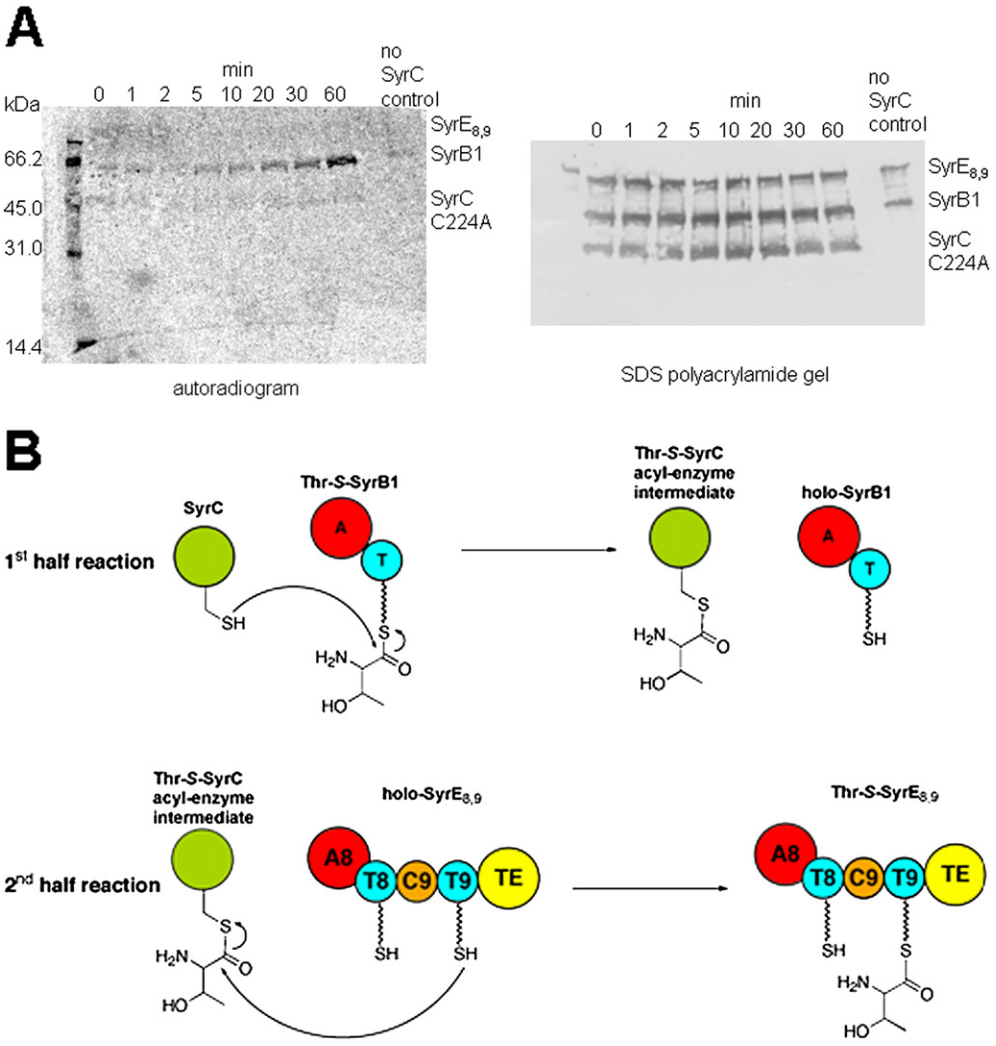

Figure 4. Assay of the SyrC C224A Mutant and Proposed Acyltransfer Mechanism

(A) Assay for L-[^{14}C]Thr transfer from SyrB1 to SyrE with the SyrC C224A mutant.

(B) Scheme depicting the two proposed half reactions involved in L-Thr transfer from SyrB1 to $SyrE_{8,9}$ via SyrC.

a time- and SyrC-dependent fashion was detected. This result shows that SyrC can both use an alternate aminoacyl moiety and recognize an alternate T domain donor scaffold when it mediates the transfer of L-[^{14}C]Val from CmaA to $SyrE_{8,9}$.

Analogous logic was used to evaluate the ability of SyrC to transfer a leucyl group from an L-[^{14}C]Leu-*S*-T domain. In this case, we had to turn to the barbamide cluster [10, 11] using the free-standing adenylation domain BarD and its cognate 10 kDa T domain BarA, in posttranslationally primed holo form. Purified BarD was used to adenylate and load L-[^{14}C]Leu onto holo-BarA. Next, SyrC was incubated with L-[^{14}C]Leu-*S*-BarA and $SyrE_{8,9}$, and the reaction was monitored as described above. Again, a SyrC- and time-dependent build-up of radioactive label on SyrE was observed, adding Leu and Val to Thr as shuttling aminoacyl groups for SyrC action (data not shown).

Using an autoradiographic assay, we found that SyrC is capable of transferring L-[^{14}C]-Thr from SyrB1 to the stand-alone T_9 domain. When similar experiments were done to determine whether SyrC can mediate the transfer of L-[^{14}C]Thr from SyrB1 to the alternate acceptor T domain, CmaD from the coronamic acid pathway [4], no transfer was observed, perhaps suggesting more stringent recognition of the T domain scaffold in its acceptor mode. In future experiments, we will carry out an accurate kinetic comparison between various T_8 and T_9 constructs, embedded and excised, along with comparison of other excised domains from the nine-module SyrE protein.

Examination of SyrC Acyltransferase Specificity for the T_8 versus T_9 Domain of SyrE

Our hypothesis that SyrC acts in *trans* to load L-Thr onto T_9 of SyrE raises the question of whether SyrC is specific enough to distinguish T_9 from all of the other T domains

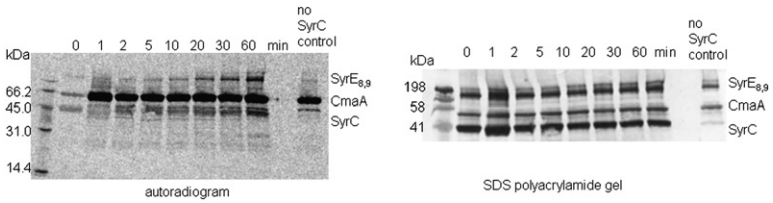

Figure 5. SyrC-Mediated Transfer of Valine to SyrE

Autoradiogram and SDS polyacrylamide gel showing SyrC-mediated transfer of L-[^{14}C]Val from CmaA to SyrE.

in the megadalton, nine-module SyrE. Numerous attempts at site-directed mutagenesis in T_8 and T_9 of the 160 kDa $SyrE_{8,9}$ fragment were attempted, but due to the large size and high GC content of the SyrE construct, we have not been able to successfully generate the mutant constructs.

Therefore, an alternate limited proteolysis approach was taken to probe SyrC specificity for T_8 versus T_9. SyrE was loaded with L-[^{14}C]Thr via SyrB1 and SyrC as described above and then subjected to cleavage at the unique thrombin site directly preceding the T_9 domain of SyrE (Figure 6A). The resulting fragments were analyzed by SDS-PAGE and autoradiography. Cleavage of the $SyrE_{8,9}$ construct with thrombin results in the formation of two fragments: $A_8T_8C_9$ (118 kDa) and T_9TE (42 kDa). If SyrC were to specifically transfer L-[^{14}C]Thr only to the T_9 domain, and not to the T_8 domain, only the 42 kDa band should be radiolabeled in the autoradiogram (Figure 6B). However, both the 118 kDa and 42 kDa bands are radiolabeled, suggesting that the SyrC aminoacyltransferase does not distinguish between the two domains in this transfer experiment. Future kinetic studies may reveal if there are rate differences for Thr transfer to the pantetheinyl arms of T_8 versus T_9.

Since aminoacylation of T_8 via SyrC would jam the syringomycin assembly line, it is possible that T_8 is aminoacylated in *cis* via A_8 much faster than it is aminoacylated in *trans* via SyrC. This would prevent SyrC from misloading T_8 with threonine. To test this hypothesis, we repeated the thrombin proteolysis assay described above, but this time added nonradiolabeled L-Asp to the reaction mixture. In this case, if L-Asp were activated and loaded onto T_8 at a greater rate than SyrC could load L-[^{14}C]Thr in *trans*, we would expect to see radiolabel only on the T_9TE fragment since the loading of nonradiolabeled L-Asp would preclude misloading of L-[^{14}C]Thr on T_8. However, we again observed radiolabel on both the $A_8T_8C_9$ and the T_9TE fragments, suggesting that there is some other mechanism of regulation that we cannot replicate in vitro.

Determination of SyrE-A_8 Substrate Specificity by ATP-PP_i Exchange Assay

The eighth amino acid in the mature syringomycin lipopeptidolactone is L-*threo*-3-OH-Asp. The five-domain $SyrE_{8,9}$ has the A_8 domain intact and thus could be tested to determine whether L-*threo*-3-OH-Asp is activated and loaded onto T_8, or whether L-Asp is loaded and hydroxylation occurs later. The aminoacyl-AMP half reaction of the A_8 domain was assayed by amino acid-dependent ATP-PP_i exchange assay [12]. At a substrate concentration of 10 mM, A_8 in the $SyrE_{8,9}$ 160 kDa fragment was able to activate L-Asp with a k_{obs} of 18.6 min^{-1}, whereas it was able to activate L-*threo*-3-OH-Asp with a k_{obs} of 0.98 min^{-1}, suggesting that hydroxylation of Asp occurs on or after the assembly line. *N*-acetyl-L-Asp and the syringomycin octapeptide comprising residues 1–8 did not support the ATP-PP_i exchange, consistent with a lack of recognition by the A_8 domain.

Condensation of L-[^{14}C]Thr with the Syringomycin Octapeptide Catalyzed by C_9

Given that $SyrE_{8,9}$ could load Asp onto T_8 and that SyrC, in the presence of SyrB1, could transfer Thr to T_9, the question of whether the C domain in module nine could catalyze peptide bond formation arose. It did not yield detectable amounts of Asp-Thr dipeptide, so we postulate

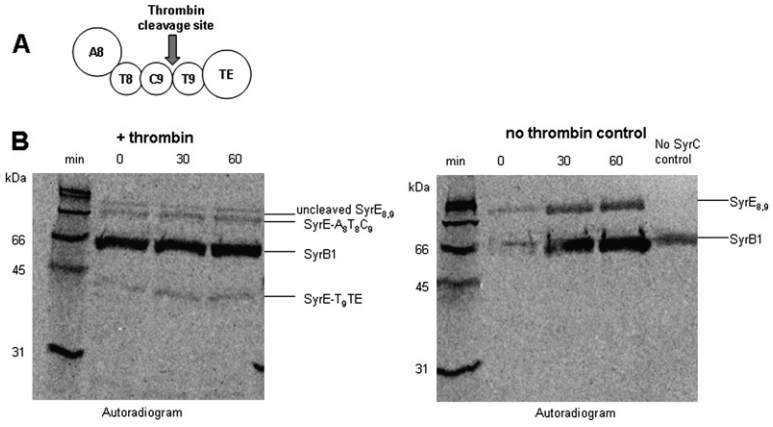

Figure 6. Thrombin Cleavage of Loaded SyrE to Determine Whether SyrC Shows Specificity for T_8 versus T_9 Domains of SyrE

(A) Cartoon diagram showing the site of thrombin cleavage.

(B) Autoradiograms of loaded SyrE after cleavage with thrombin (left), and no thrombin control (right).

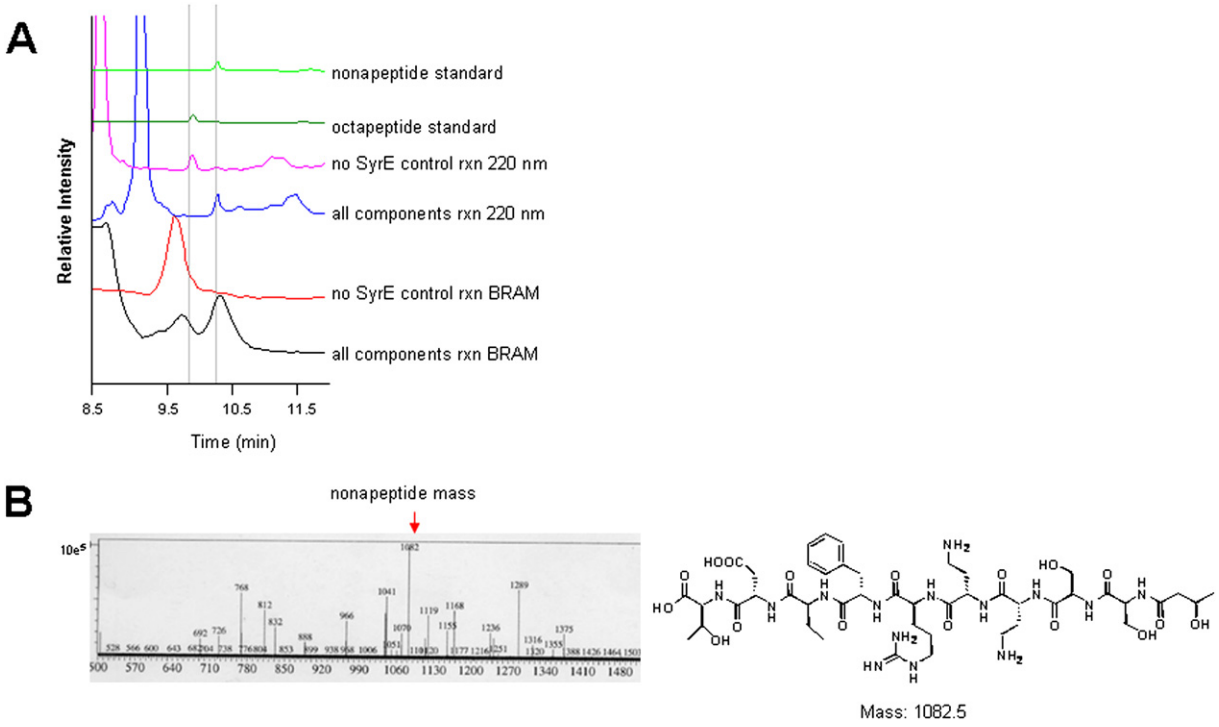

Figure 7. Generation of the Syringomycin Nonapeptide on SyrE

(A) HPLC chromatogram indicating products of the nonapeptide formation reaction and control reactions.

(B) LC-MS spectrum of reaction products that elute at 2.4 min, the retention time of the nonapeptide standard on the LC-MS column.

that the amino group of Asp may need to be acylated or, perhaps, the full-length lipooctapeptidyl chain needs to be presented. To test this hypothesis, we attempted to assay for the condensation of an octapeptide substrate with L-Thr. We and then others [13] have previously reported the synthesis of peptidyl CoAs and their loading onto apo forms of T domains catalyzed by Sfp to yield peptidyl-*S*-T domains. In the case of $SyrE_{8,9}$, when β-OH-butyryl-octapeptidyl CoA was incubated with Sfp and apo $SyrE_{8,9}$ to load either T_8 or T_9 stochastically, and then holo SyrB1, SyrC, ATP, and Thr were added, nonapeptide formation was assayed. Condensation was allowed to proceed for 1 hr, then the protein was precipitated and pelleted and the supernatant was analyzed for nonapeptide product by radio-HPLC and LC-MS. The radio-HPLC chromatogram of the supernatant shows the formation of a new peak with the same retention time as the nonapeptide standard, and there is a corresponding peak in the 220 nm UV chromatogram. This peak is not apparent in the absence of SyrE (Figure 7A). The LC-MS spectrum of the reaction mixture shows a mass corresponding to that of the nonapeptide, with the same retention time as the nonapeptide standard (2.4 min). Again, this mass is absent when SyrE is not present in the reaction mixture (Figure 7B). These preliminary data suggest that the C_9 domain catalyzes condensation of octapeptidyl substrate on T_8 with L-[^{14}C]Thr on T_9 to produce and release the linear syringomycin nonapeptide.

Subsequent efforts will determine to what extent the chain length of the β-OH-acyl substituent controls hydrolytic versus macrolactonizing release.

Formation of Syringomycin Nonapeptide Analogs Catalyzed by C_9

Since SyrC is capable of loading alternate amino acids, such as L-Val and L-Leu, onto SyrE, it is possible that the C_9 domain of SyrE could catalyze the condensation of these amino acids with the octapeptidyl substrate to generate novel syringomycin nonapeptides. Using the same methods as described above, we loaded SyrE with octapeptide and then loaded either L-Leu or L-Val onto SyrE via SyrC. Analysis by LC-MS indicates that small amounts of the alternate peptides are formed, and the low yield of the alternate peptides suggests that C_9 may not readily accept the alternate amino acids loaded onto T_9 (Figure S4).

DISCUSSION

The assembly line for the nonribosomal nonapeptidolactone phytotoxin syringomycin from *Pseudomonas syringae* has been a mystery since the report of the *syr* biosynthetic gene sequence—*syrB1*, *syrB2*, *syrC*, *syrE*—and the deduced domain organization by Guenzi et al. in 1998 [1]. Notable among the mysteries was the fact that the nine-module SyrE was missing the A domain in module nine,

suggesting that the MDa protein SyrE would be incompetent to activate and load the terminal residue, Thr_9 or 4-Cl-Thr_9, to finish chain elongation. Further, the A domain in the free-standing A-T didomain enzyme SyrB1 was able to activate Thr. This suggested that SyrB1 was in effect the "missing" A_9, and that the Thr_9 must come in *trans* from SyrB1 to module nine of SyrE each time a syringomycin chain is completed on the SyrE assembly line. We clarified part of the puzzle by recently demonstrating the function of SyrB2 as a novel mononuclear nonheme Fe^{II} halogenase that acts on Thr-*S*-SyrB1 to produce 4-Cl-Thr-*S*-SyrB1. This is the catalytic step generating the Cl-Thr_9 donor on the pantetheinyl arm of SyrB1 [3].

In the present work, we demonstrate the function of the remaining Syr protein, the 44 kDa SyrC, and validate the in *trans* shuttling of Thr/Cl-Thr from SyrB1 to SyrE. When the sequence of SyrC was reported, it was clearly a member of the α/β-hydrolase superfamily. Postulated functions included thioesterase, haloperoxidase, or perhaps an acyltransferase for the N-terminal β-OH-fatty acyl moiety [1, 6]. We have previously assayed SyrC for such *N*-acylation activity of Ser_1-*S*-SyrE without any success (R.G. Kruger, F.H.V., C.T.W., data not shown).

Two parallel efforts on other NRPS systems in which aminoacyl/acyl moieties are shuttled gave us some inkling of the function of SyrC demonstrated in this work. One was in the coronamic acid biosynthetic pathway in which the free-standing A-T didomain CmaA corresponds to SyrB1 and the nonheme iron halogenase CmaB corresponds to SyrB2 [4]. CmaE, a homolog of SyrC, shuttles an L-*allo*-Ile moiety between the pantetheinyl arm of the T domain of CmaA and the free-standing T domain of CmaD. Only when the aminoacyl group is presented as L-*allo*-Ile-*S*-CmaD will the CmaB halogenase recognize it [4]. CmaE is thus an aminoacyltransferase that shuttles the *allo*-Ile moiety between holo T domain scaffolds.

The second example is the recent demonstration that the last step in coumermycin and clorobiocin biosynthesis involves transfer of the acyl group, pyrrole-2-carbonyl, from the pantetheinyl arm of a T domain to the 3′-OH of the noviosyl ring of the antibiotic [5]. CmaE uses an active site Cys as a nucleophile, while CouN7 uses an active site Ser as a nucleophile, to make acyl-*S*- and acyl-*O*-enzyme intermediates, respectively, to shuttle the substrate acyl fragments between acceptors. In this context, SyrC joins this family of acyl/aminoacyl-*S*-pantetheinyl-T domain shuttle enzymes and solves the need for an in *trans* delivery of an activated Thr/Cl-Thr moiety to module nine of SyrE.

The assay of SyrC activity was not entirely straightforward. The protein scaffold for the aminoacyl donor is the 66 kDa holo form of the SyrB1 protein, which can be made in multimilligram quantities. The 4-Cl-Thr amino acyl moiety is not available in radioactive form, cannot be loaded by the A domain of SyrB1 [3], and is not quantitatively available by action of SyrB2 on Thr-*S*-SyrB1. Therefore, we used L-[^{14}C]Thr as a surrogate for Cl-Thr. The acceptor substrate is even harder to come by: *P. syringae* SyrE has a molecular weight of 1,038,663 Da and is not readily expressable in *E. coli*. Of various fragments of SyrE, we chose the 160 kDa A_8-T_8-C_9-T_9-TE (deemed $SyrE_{8,9}$) to express and purify in soluble form to evaluate various aspects of the last step of syringomycin assembly. In principle, $SyrE_{8,9}$ could allow assay of in *trans* import of L-[^{14}C]Thr from SyrB1, loading of Asp/β-OH-Asp by A_8 on T_8, condensation activity of C_9, and release activity of the TE domain. In this study, we have focused mostly on demonstrating SyrC's aminoacyl shuttle activity.

The SyrC activity assay devolved to monitoring L-[^{14}C]Thr transfer from L-[^{14}C]Thr-*S*-SyrB1 to holo $SyrE_{8,9}$. Our hypothesis that SyrC, like CmaE and CouN7 [4, 5], may engage in covalent catalysis is confirmed by autoradiography of SyrC in SDS-PAGE studies, with the thiolate side chain of Cys224 as the likely catalytic nucleophile. Thus, the shuttle enzymes CmaE and SyrC carry out energetically neutral aminoacyl transfers between thiolates of pantetheinyl prosthetic groups by way of a covalent thioester linkage in the shuttling enzyme. In contrast, CouN7, which uses a Ser-OH nucleophile, catalyzes the energetically favorable transfer of the pyrrole-2-carboxy acyl group from a pantetheinyl thioester in CouN1 to an oxoester in the pyrrolyl-coumarin antibiotic scaffold [5].

The $SyrE_{8,9}$ fragment is not ideal for determination of the T domain selectivity of SyrC, but it appears that the itinerant L-[^{14}C]Thr can end up on the pantetheinyl forms of both T_8 and T_9 of this enzyme fragment. Whether occupancy of T_8 by the waiting octapeptidyl chain is a default director of the incoming Thr to T_9, or there are higher-order conformer constraints in the full-length, million molecular weight SyrE is yet unknown. Presumably, transfer of the L-[^{14}C]Thr/Cl-Thr to an empty holo-T_8 would jam the assembly line and, thus, would have to be avoided or hydrolytically released. In subsequent efforts, we shall begin to address the action of C_9 and the TE domain, perhaps in a smaller C_9-T_9-TE construct, to begin to study the affinity of SyrC for SyrE T domains, the kinetics and specificity of transfers of the aminoacyl moiety that will become residue 9, and the permissivity of the C_9 condensation step and macrolactonization.

Given our recent detection of the three examples noted, the question of how frequently aminoacyl shuttle enzymes will turn up in NRPS assembly lines arises. They should allow in *trans* aminoacyl group insertions to create diversity at particular sites. If different shuttling enzymes recognize distinct sets of T domains, then it may become possible to increase positional diversity in nonribosomal peptides in their presence.

SIGNIFICANCE

The final step of the syringomycin biosynthetic assembly line, which involves the incorporation of chlorothreonine into the syringomycin scaffold, has long been a mystery due to the fact that SyrE, the megadalton syringomycin synthetase, lacks an adenylation domain in its ninth module. Prior work has shown that chlorothreonine is generated by the action of the

Fe^{II}/α-ketoglutarate-dependent halogenase SyrB2 on a threonine residue that is tethered via a phosphopantetheinyl linkage to the A-T didomain protein SyrB1. Here, we demonstrate that SyrC is an acyltransferase that is capable of shuttling threonine from SyrB1 to SyrE. Furthermore, we present preliminary data suggesting that the ninth condensation domain of SyrE is capable of catalyzing the condensation of threonyl-*S*-SyrE-T_9 with an octapeptidyl substrate to generate a linear nonapeptide, which is then hydrolyzed by the thioesterase domain. We also show that SyrC is capable of transferring alternate amino acids, such as leucine and valine, onto SyrE, and that these amino acids can then be incorporated into a linear nonapeptide. These results suggest that SyrC may be useful in the combinatorial enzymatic synthesis of syringomycin analogs, and they set the stage for further studies into the final steps of syringomycin biosynthesis. In addition, our identification of SyrC as an aminoacyltransferase adds another member to the small but growing family of NRPS/PKS acyltransferases that use covalent catalysis to shuttle aminoacyl groups between carrier proteins. This family of aminoacyltransferases may be useful in creating positional diversity in nonribosomal peptides.

EXPERIMENTAL PROCEDURES

Materials and General Methods

All radiolabeled chemicals were obtained from American Radiolabeled Chemicals, Inc. (ARC). L-*threo*-3-OH-Asp was obtained from Tocris Pharmaceuticals. All other chemicals used were from Sigma, unless otherwise specified. The TOP10- and BL21(DE3)-competent *E. coli* strains were purchased from Stratagene. The *Pfu*Turbo DNA Polymerase used in PCR was purchased from Stratagene. During protein purification, cells were lysed with an Avestin Emulsiflex-C5 cell disruptor. Thrombin was obtained from Novagen. SDS polyacrylamide gels were obtained from BioRad, and autoradiography was performed on a Typhoon 9400 scanner (GE Healthcare). HPLC was carried out on a Beckman Coulter System Gold by using a Vydac C18 column. Radio-HPLC was performed on an identical system equipped with a β-Ram radioisotope detector (IN/US). LC-MS analysis was performed on an LCMS-QP8000a spectrometer (Shimadzu) with a Vydac C18 LC-MS column. Recombinant BarA and BarD were provided by Danica Galonic, and recombinant CmaA was provided by Eric Strieter.

Cloning of Syr Genes

SyrB1 was cloned as described [3]. The SyrC and $SyrE_{8,9}$ constructs were obtained by PCR amplification of genomic DNA from *Pseudomonas syringae* pv. *syringae* B301D, which was a gift from Dennis C. Gross (Texas A&M University, College Station, Texas) [14]. *P. syringae* pv. *syringae* was grown in nutrient broth-yeast extract medium at 30°C, and genomic DNA was isolated by using the Bactozol kit for bacterial DNA extraction (Molecular Research). Amplification was carried out by using *Pfu*Turbo DNA Polymerase in accordance with the manufacturer's instructions. The following oligonucleotide primers were used in PCR amplification: SyrC: 5′-GGAATTCCATATGCGCGTTTG CGGCATT-3′ and 5′-CCCAAGCTTCATCATGGGAAGCTGGGACA-3′; $SyrE_{8,9}$: 5′-CGGAATTCACTACTCACTGGCGCGGT-3′ and 5′-GGAAT TCCATATGCTTGAGCAGGATCCGGCA-3′. The SyrC PCR product was digested with NdeI and HindIII, and the $SyrE_{8,9}$ construct was digested with NdeI and EcoRI. All digested PCR products were ligated into similarly digested pET28b plasmids to create N-terminally His_6-tagged constructs.

Overexpression and Purification of Syr Proteins

The pET-28a expression vectors containing the Syr proteins were transformed into BL21 (DE3)-competent cells. Cultures were grown in Luria-Bertani medium supplemented with 30 μg/ml kanamycin at 37°C until the OD_{600} reached ~0.3, at which time the cultures were cooled to 25°C and grown until the OD_{600} reached ~0.6. The cultures were induced with 0.1 mM IPTG and were grown at 15°C overnight. Cells were harvested by centrifugation at 6,000 rpm for 30 min, flash frozen in liquid N_2, and stored at −80°C until further purification.

Cells were thawed, resuspended in Buffer A (300 mM NaCl, 5 mM imidazole, 20 mM Hepps [pH 8.0]), and lysed by cell disruption. Cell debris was removed from the lysate by centrifugation at 15,000 rpm for 30 min, and the supernatant was removed and bound to Ni-NTA resin by rocking at 4°C for 2 hr. The resin was added to a Bio-Rad Econo-Sphere column and washed with Buffer A. Protein was eluted with Buffer B (300 mM NaCl, 30 mM imidazole, 20 mM Hepps [pH 8.0]) and Buffer C (100 mM NaCl, 200 mM imidazole, 20 mM Hepps [pH 8.0]). Protein-containing fractions were identified by SDS-PAGE, combined, and dialyzed overnight in 100 mM NaCl, 1 mM EDTA, 50 mM Hepps (pH 8.0) with 10% glycerol. The dialyzed protein was concentrated, flash frozen in liquid N_2, and stored at −80°C.

Site-Directed Mutagenesis of SyrC

The pET28a plasmid containing the *syrC* gene was used as the template for splicing by overlap extension (SOE) to generate the C224A mutant protein. The standard two-step SOE PCR method was used to create a mutation at the desired site [15]. In the first round of PCR, the 5′ end of the SyrC protein was amplified with the following primers: 5′-CGGGGATCCCATATGACTATTTCCTCCGAT-3′ (forward) and 5′-GCC**GGC**GATGCCCATCAGATGTGCGGTCGA-3′ (reverse internal). Also in the first round of PCR, the 3′ end of the SyrC protein was amplified with the following primers: 5′-CGGATAGAGCTCT CAGGCGACAGCGGGCTG-3′ (reverse) and 5′-CATCTGATGGGCAT C**GCC**GGCGGCGCGGTCATC-3′ (forward internal). Underlined bases indicate restriction sites, and bold base pairs indicate the sites of mutation. The two resulting fragments were purified by using the Qiagen PCR Purification kit and were then mixed and used in the second round of PCR with the forward and reverse primers from the first step. The resulting PCR product was digested with XhoI and NdeI and ligated into the pET28a plasmid. The presence of the mutation in the resulting vector was verified by sequencing. The SyrC C224A mutant protein was expressed and purified as described above.

Generation of Phosphopantetheinlyated Enzymes

Recombinant enzymes containing thiolation domains, such as SyrB1, SyrE, CmaA, and BarA, must be phosphopantetheinlyated prior to their use in assays. In a typical reaction, 1 nmol enzyme is incubated with $MgCl_2$ (0.5 μmol), CoA (50 nmol), Sfp (10 nmol) in 50 mM HEPES buffer (pH 7.5), in a total reaction volume of 25 μl for 30 min at room temperature to produce the holo enzyme.

Solid-Phase Synthesis of Octapeptide, COOH-L-Asp-L-Abu-L-Phe-L-Arg-L-Dab-D-Dab-D-Ser-L-Ser-N-Ac, and Nonapeptide, COOH-L-Thr-L-Asp-L-Abu-L-Phe-L-Arg-L-Dab-D-Dab-D-Ser-L-Ser-N-Ac

Peptide synthesis was performed with a PerSeptive Biosystems 9050 synthesizer (0.3 mM scale) by using diisopropylcarbodiimide (DIPCDI)/hydroxybenzotriazole (HOBt) chemistry. The peptide was cleaved from the resin, deprotected in a single treatment with 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropylsilane (TIS) at room temperature for 3 hr. The solution was then added to cold ether dropwise, and the precipitated peptide was collected by centrifugation. The purified peptides (Figure S3) were lyophilized to yield a white powder, and the identity and purity were established by analysis on LCMS.

Synthesis of Coenzyme A Derivatives of Octapeptide and Nonapeptide

The peptidyl-CoA derivatives (Figure S3) were prepared based on a previously reported protocol [16]. The peptide was cleaved from the resin with 1:1:3 acetic acid:trifluoroethanol (TFE):dichloromethane (DCM) and was incubated at room temperature for 3 hr. The resin was removed by filtration, and n-hexane was added to precipitate the fully protected peptide. After rotary evaporation to remove the solvent, the peptide was redissolved in DCM and precipitated with n-hexane; this was repeated twice. The CoA coupling to the protected peptide was accomplished by the addition of 1 equivalent of CoA (Li+ salt; Sigma), 4 equivalents of potassium carbonate, and 1.5 equivalents of PyBOP in 1:1 THF:water. The reaction was mixed by tipping for 2 hr at room temperature, and then the solvent was removed by rotary evaporation followed by lyophilization. Removal of the N-terminal Boc-protecting groups was achieved by treatment with 95% TFA, 2.5% water, and 2.5% TIS at room temperature for 3 hr. The solution was then added to cold ether dropwise, and the precipitate was removed by centrifugation after overnight incubation at −20°C. The peptidyl-CoA was then dissolved in acetonitrile/water and purified by preparative HPLC on a reverse-phase C18 column with a gradient of 0%–100% acetonitrile in 0.1% TFA/water over 35 min. The pure peptidyl-CoA had a retention time of 16 min. The purified compounds were lyophilized, and the identity and purity were established by analysis in negative ion mode on LCMS and MALDI-TOF mass spectrometry: octapeptidyl-CoA 1711.5 [$(M - H)^-$] calculated, 1711.1 observed; nonapeptidyl-CoA 1811.6 [$(M - H)^-$] calculated, 1811.7 observed.

Assay for Transfer of L-[^{14}C]Thr from SyrB1 to SyrC

Holo-SyrB1 (0.4 nmol) was incubated with L-[^{14}C]Thr (9 nmol) and ATP (200 nmol) in 50 mM HEPES buffer (pH 7.5), in a total reaction volume of 62 μl for 10 min at room temperature to generate L-[^{14}C]Thr-*S*-SyrB1. To this reaction mixture, SyrC (0.6 nmol) was added, and 8 μl aliquots of the reaction were quenched in 2× SDS-PAGE loading buffer (without reducing agent) at various time points. The quenched aliquots were heated to 70°C for 10 min and then run on a 12% SDS polyacrylamide gel. The gel was stained, destained, dried, and exposed to a phosphorimager screen for 3 days, after which the screen was scanned with a Typhoon imager.

Assay for SyrC-Mediated Transfer of [^{14}C]-Labeled Amino Acids from SyrB1 to SyrE

In a typical reaction (66 μl), holo-SyrB1 (0.4 nmol) and holo-SyrE$_{8,9}$ (0.2 nmol) were incubated with L-[^{14}C]Thr (9 nmol) and ATP (200 nmol) in 50 mM HEPES (pH 7.5). Recombinant SyrC (0.6 nmol) was added to initiate the reaction, and time points were quenched and run on a gel as described above. In tandem, a reaction lacking SyrC was performed as a negative control. The gels were processed as described above. Similar reactions to examine the ability of SyrC to transfer alternate amino acids were carried out with L-[^{14}C]Leu and L-[^{14}C]Val, with donor T domains BarA and CmaA, respectively.

Assay for Formation of the Syringomycin Nonapeptide on SyrE$_{8,9}$

Holo-SyrE$_{8,9}$ (1.4 nmol) was incubated with ATP (100 nmol) and octapeptidyl-*S*-CoA (20 nmol) in 50 mM HEPES (pH 7.5), in a total reaction volume of 45 μl for 1 hr at room temperature. The octapeptidyl-*S*-SyrE was then incubated with holo-SyrB1 (2.8 nmol), L-[^{14}C]Thr (9 nmol), and ATP (450 nmol) in HEPES. Recombinant SyrC (4.2 nmol) was added to initiate the reaction (140 μl total volume), which was incubated at room temperature for 1 hr to allow nonapeptide formation to proceed. In tandem, reactions lacking peptide, L-[^{14}C]Thr, SyrB1, SyrC, or SyrE were carried out as controls.

The reactions were quenched in 100 μl 10% (v/v) trichloroacetic acid (TCA) to precipitate the proteins. The proteins were pelleted by centrifugation at 13,000 rpm for 20 min, and the supernatant was removed and saved (supernatant A). The pellet was washed twice with 100 μl 10% TCA and then redissolved in 100 μl 0.1M LiOH and heated to 85°C to release any peptide still bound to the SyrE$_{8,9}$ scaffold. The protein was then reprecipitated by the addition of 20 μl 50% TCA and pelleted by centrifugation, and the supernatant was removed and saved (supernatant C).

The two supernatants from each reaction were analyzed by HPLC and LC-MS to determine if they contained the radiolabeled nonapeptide product. The HPLC analysis was carried out by using a Vydac C18 small-pore column with a water/acetonitrile gradient going from 0%–100% acetonitrile over 30 min. HPLC was monitored both for absorbance at 220 nm and for ^{14}C radioactive counts. By LC-MS, the nonapeptide was observed in supernatant A of the reaction containing all components: 1083.1 [$(M + H^+)$] calculated, 1082.0 observed.

ATP-PP$_i$ Exchange Assays to Determine SyrE-A$_8$ Substrate Specificity

Each reaction contained 10 mM amino acid, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 2 μM SyrE$_{8,9}$, and 5 mM sodium [^{32}P]pyrophosphate in a total volume of 500 μl with 50 mM HEPES (pH 7.5). In tandem, reactions containing no enzyme were carried out as negative controls. Reactions were initiated by the addition of enzyme, and aliquots were quenched at 0, 1, 2, 5, 10, 20, 30, and 60 min by the addition of 750 μl of a solution containing 1.6% (w/v) activated charcoal, 200 mM sodium pyrophosphate, and 3.5% (v/v) perchloric acid. For each time point, the charcoal was pelleted by centrifugation and washed twice with 750 μl of a solution containing 200 mM sodium pyrophosphate with 3.5% perchloric acid (wash buffer). The charcoal pellet was then resuspended in 750 μl wash buffer and mixed with liquid scintillation fluid. Radioactivity bound to the charcoal was measured by liquid scintillation counting.

Assay for SyrC Specificity for SyrE$_{8,9}$ T Domains by Thrombin Cleavage

To determine whether SyrC can specifically transfer L-[^{14}C]Thr from SyrB1 to the T$_9$ domain of SyrE, or whether it transfers the amino acid to both T$_8$ and T$_9$, holo-SyrE$_{8,9}$ (0.2 nmol) was loaded with L-[^{14}C]Thr via SyrC and SyrB1 as described above in a total reaction volume of 78 μl. Time points were collected at 0, 30, and 60 min, by flash freezing 20 μl aliquots of the reaction in liquid nitrogen. Then, the aliquots were thawed, and 10 μl of each aliquot was incubated with 0.1 units of thrombin in thrombin-cleavage buffer for 3 hr at 22°C. The remaining 10 μl of each aliquot was incubated under the same conditions in thrombin-cleavage buffer without the addition of thrombin as a negative control. Samples were run on a 12% SDS polyacrylamide gel, and the gel was processed as described above.

Supplemental Data

Supplemental Data include four figures and are available at http://www.chembiol.com/cgi/content/full/14/1/31/DC1/.

ACKNOWLEDGMENTS

We thank Danica Galonic and Eric Strieter for providing BarA/BarD and CmaA, respectively, as well as for many helpful discussions. This work was supported in part by National Institutes of Health grant GM20011 (C.T.W.), a National Science Foundation Predoctoral fellowship (G.M.S), a Merck-sponsored fellowship of the Helen Hay Whitney Foundation (F.H.V.), and a Natural Sciences and Engineering Research Council of Canada Postdoctoral fellowship (F.H.V).

Received: August 14, 2006
Revised: October 27, 2006
Accepted: November 3, 2006
Published: January 26, 2007

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